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In Vitro and *in Vivo* Characterization of a Second Functional Hairpin Ribozyme against HIV-1

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We have constructed a hairpin ribozyme targeted to cleave a conserved sequence in the HIV-1 *pol* gene. The ribozyme was modified to include a structure-stabilizing tetraloop. *In vitro* studies revealed a cleavage efficiency unprecedented for hairpin ribozymes (k_{cat}/K_m = 75 min^{-1} μM^{-1}). Stable retroviral vector transduction of this ribozyme gene in T-cell lines resulted in long-term ribozyme expression. As compared to control vector transduced T-cells, the pol ribozyme-transduced cells exhibited significant inhibition of different strains of HIV-1 virus production; this protection was greater when ribozyme expression was driven from an internal pol III promoter (adenovirus VA1) than when driven by a pol II promoter (the MMLV LTR). These results further demonstrate the potential of hairpin ribozymes as anti-HIV gene therapy agents and suggest possibilities for employing combinations of independently targeted hairpin ribozymes.

INTRODUCTION

Because of the ability of ribozymes to bind to and cleave viral substrate RNAs (Cech *et al.,* 1981; Guerrier-Takada *et al.,* 1983), these catalytic RNA molecules are being adapted to antiviral gene therapy, specifically, anti-HIV gene therapy (Sarver *et al.,* 1990). The catalytic properties of ribozymes give them a potential advantage over antisense RNAs (Rossi *et al.,* 1992). Both hammerhead and hairpin ribozymes, which derive their names from their characteristic secondary structures, are small molecules that can be readily engineered to cleave target RNA with high efficiency and specificity (Forster and Symons, 1987; Uhlenbeck, 1987; Hampel *et al.,* 1990). Both have been shown to specifically recognize and cleave HIV-1 RNA transcripts and thereby inhibit HIV-1 gene expression (for reviews see Altman, 1993; Yu *et al.,* 1994a).

The hairpin ribozyme is a 50- to 60-nt catalytic RNA molecule that can be designed to bind and efficiently cleave heterologous RNA molecules in a highly specific manner (Hampel *et al.,* 1990) as long as simple targeting rules are followed (Hampel *et al.,* 1993; Anderson *et al.,* 1994). To summarize, the ribozyme recognizes 5′NNNB N*GUC NNNNNN . . . N3′ sequence in the target RNA with base-pairing occurring between the ribozyme and the substrate in the two sequences flanking the N*GUC (* denotes the cleavage site). N can be any nucleotide and B can be U, C, or G, but not A. We have reported previously that a hairpin ribozyme targeting the 5′ leader sequence of HIV-1 is highly effective in inhibition of viral gene expression and replication (Ojwang *et al.,* 1992; Yu *et al.,* 1993; Yamada *et al.,* 1994). When the ribozyme was delivered via retroviral vectors and driven by the vector LTR or by pol III promoters, reduction of infectious progeny virus was $>10^4$-fold, expression of viral proteins was inhibited 100- to 1000-fold, and the synthesis of viral DNA was reduced by 50- to 100-fold in stably transduced T-cell lines. Moreover, this ribozyme was highly effective against HIV-1 in primary cells, including peripheral blood lymphocytes (Leavitt *et al.,* 1994) and monocytes/macrophages derived from transduced CD34+ hematopoietic stem/progenitor cells (Yu *et al.,* 1994b).

In this study we have identified and tested a new hairpin ribozyme targeted to a highly conserved region in the pol gene of HIV-1. This ribozyme has a modified sequence in loop 3 and helix 4 to increase stability. The sequence recognized and cleaved is 5′CACC U*GUC AACAUAA3′. *In vitro* studies showed high cleavage efficiency, with a k_{cat}/K_m value of 75 min^{-1} μM^{-1}. Human T-cells transduced with retroviral vectors carrying the gene persistently expressed the ribozyme after a long period of time with no apparent deleterious effects on cell proliferation. Most importantly, the "intracellularly immunized" cells were resistant to different strains of HIV infection. The identification of an additional active ribozyme targeting HIV-1 raises the possibility of developing "multitarget" ribozyme vectors to increase antiviral potency and minimize the possibility of emergence of resistant variants.

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MATERIALS AND METHODS

Design and development of the hairpin ribozyme

The site. The HIV-1 pol-specific hairpin ribozyme was designed to cleave the 5′CACC U*GUC AACAUAA3′ target sequence (nt 2490–2504) in the protease region of the pol gene of HIV-1 (Fig. 1). Numbering is according to the HXB2 clone of HIV-1 (Ratner *et al.,* 1987). This sequence was identified by searching for highly conserved GUC containing sequences in GenBank using the Bioseq program (Aspinall). As shown in the alignment below, this target sequence is highly conserved among different HIV-1 strains; HIV-1 Eli, which has a single A → G substitution at position 2 is the only exception (see boldface underlined characters) among all the published HIV-1 sequences.

HXB-2	CA CCU GUC AAC ATA A
BRU	CA CCU GUC AAC ATA A
MN	CA CCU GUC AAC ATA A
OY1	CA CCU GUC AAC ATA A
SF2	CA CCU GUC AAC ATA A
HAN	CA CCU GUC AAC ATA A
RF	CA CCU GUC AAC ATA A
ELI	C**G** CCU GUC AAC ATA A
Z2	CA CCU GUC AAC ATA A
NDK	CA CCU GTC AAC ATA A
MAL	CA CCU GTC AAC ATA A

The ribozyme. The pol-specific hairpin ribozyme was designed to hybridize to the bases flanking the U*GUC sequence of the substrate. Hybridization forms helices 1 and 2 of 7 and 4 bp, respectively (Fig. 2). The GUU sequence found in loop 3 of the conventional hairpin ribozyme (Hampel *et al.,* 1990) was replaced by the tetraloop sequence 5′GGAC (UUCG) GUCC3′ (Fig. 2; Cheong *et al.,* 1990). Labeled pol-specific ribozyme and corresponding short substrates were produced for optimization and kinetic studies by transcription with [P^{32}]CTP from synthetic oligodeoxynucleotide templates containing the T7 promoter as previously described (Hampel and Tritz, 1989). For *in vitro* studies all ribozymes had an additional GGG vector sequence at the 5′ terminus and all substrates had GCG at the 5′ terminus to allow effective transcription from the T7 promoter.

Kinetic studies

The kinetic parameters K_m and k_{cat} were determined using the [P^{32}]CTP-labeled ribozyme and substrate sequences shown in Fig. 2 with the additional vector sequence at the 5′ end of each transcript. Cleavage of the substrate by the ribozyme was carried out in 12 m*M* $MgCl_2$, 2 m*M* spermidine, and 40 m*M* Tris, pH 7.5, at 37° as previously described (Hampel and Tritz, 1989). Reaction products and substrates were separated on 20% polyacrylamide–7 *M* urea gels in TBE buffer and counted by liquid scintillation spectrometry. Concentrations of ribozymes were from 1 to 10 n*M* and were fixed for each kinetic determination. Substrate concentrations ranged from 10 to 200 n*M* for the determination of initial velocities. K_m and k_{cat} were calculated from an Eadie–Hoffstee plot of the data as before (Hampel and Tritz, 1989).

Cloning of the Pol ribozyme gene into retroviral vectors

The Pol ribozyme gene was cloned into several retroviral vectors including those containing pol III internal promoters (Yu *et al.,* 1993). DNA corresponding to the Pol-specific ribozyme, flanked by *Bam*HI and *Mlu*1 sites, was synthesized on an ABI 381A DNA synthesizer and cloned into *Bam*HI/*Mlu*I sites of the plasmid pHC which contains the hairpin autocatalytic cassette (Altschuler *et al.,* 1992) to derive the plasmid pRPol. The retroviral vector pMM-6 was constructed by replacing the leader sequence ribozyme gene in pMJV (Yu *et al.,* 1993, 1994b) with the Pol ribozyme gene taken from pRPol between the restriction sites *Bam*HI and *Mlu*I, leaving the pol III promoter and terminator intact (Fig. 3A).

Another vector was constructed by placing the anti-pol ribozyme under the control of the MMLV LTR (pol II) promoter. In anticipation of constructing multitarget hairpin ribozyme vectors, we developed a dicistronic retroviral vector, pLPONL, containing the neogene preceded by the 5′ nontranslated region (5′NTR) of poliovirus (indicated as "PO" in Fig. 3A). Previous work showed that this 5′NTR allows internal initiation of translation to occur in mammalian cells (Pelletier and Sonenberg, 1988). pMY-1 was constructed with the anti-pol ribozyme gene including an autocatalytic cassette (Altschuler *et al.,* 1992; Ojwang *et al.,* 1992; also see construct pMJF-1 in Yu *et al.,* 1993) inserted downstream of the 5′LTR of the retroviral vector, pLPONL, at the *Hin*dIII site (Fig. 3A).

Stable cell lines and ribozyme expression

The human T-cell lines, Jurkat and Molt 4 clone 8 were stably transduced with retroviral vectors and established as previously described (Yamada *et al.,* 1994). Briefly, the retroviral vector plasmids were first transfected into the amphotropic retroviral packaging cell line PA317 by the Ca^{2+} PO_4 method. Virus supernatant was harvested 2 days after transfection and used to transduce human T-cell lines. Neoresistant (neo^r) cells were then established by G418 selection (400 μg/ml). Total RNA was extracted from the stable cell lines by the acid guanidinium thiocyanate-phenol/chloroform extraction method (Chomczynski and Sacchi, 1987). One microgram of the total RNA was used as the template for reverse transcription. RT-PCR

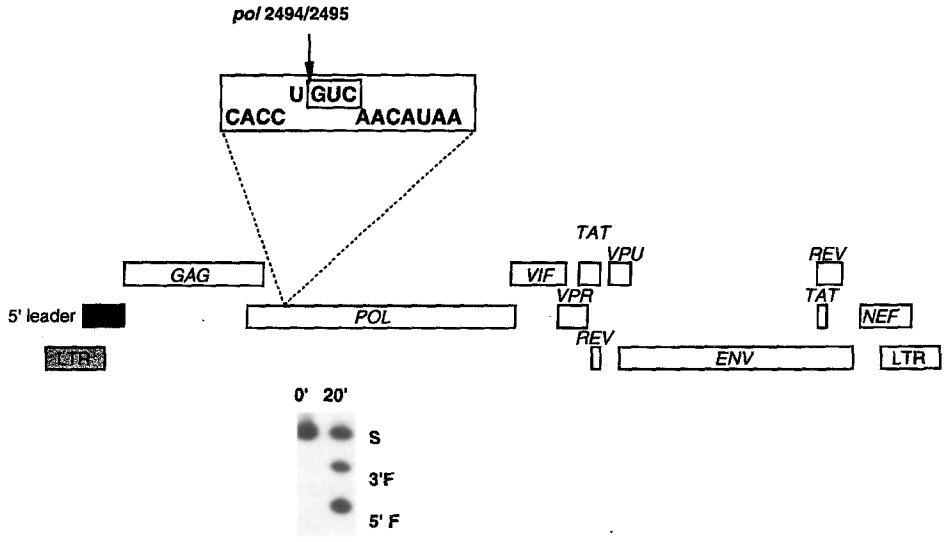

FIG. 1. The location of the target and the sequence cleaved in the pol gene of HIV-1. Numbering is according to the HXB2 clone of HIV-1 (Ratner *et al.* 1987). Shown below is cleavage of the HIV-1 pol substrate by HIV-1 pol ribozyme. In this case, the pol specific hairpin ribozyme was 0.04 μM and the pol substrate was 0.2 μM, incubated at 37° for 20 min and electrophoresed in a 20% polyacrylamide gel. Extent of cleavage was 62%.

was carried out with a 5′-end Pol ribozyme-specific primer, Rib P (18 mer, 5′-TTA TCT TAG AAG GTG ACC-3′), and the 3′-end common hairpin ribozyme primer Rib 2 (Yamada *et al.*, 1994), using conditions described previously (Yamada *et al.*, 1994). After PCR with and without RT, 10 μl of each amplified product was subjected to electrophoresis on a 3% low melting agarose gel, and Southern blot analysis was performed with another ^{32}P-end-labeled Pol ribozyme-specific probe, Rib T (24 mer, 5′-AGA GAA ACA CAC GGA CTT CGG TCC-3′).

HIV-1 virus challenge

HIV-1 HXB2 virus stocks were grown in acutely infected Molt4/8 T-cells. The infectious titer ($TCID_{50}$) was determined using the MT-2 cell line by the standard method of limiting dilution. Jurkat and Molt 4/8 cells stably expressing the Pol ribozymes and LNL6-transduced control cells were synchronously passaged for 2 weeks prior to challenge to ensure that all cell lines were challenged under the same growth conditions. Cells were infected at different m.o.i.s of 0.01 and 0.001. The cells were washed twice with RPMI 1640 medium after virus-adsorption for 4–12 hr and resuspended in RPMI 1640 supplemented with 10% FBS at a concentration of 10^5 cells/ml. During the cell culturing period, small aliquots of the culture fluid were collected every 2 days, and the level of HIV-1 p24 antigen was determined by HIV-1 antigen capture ELISA test (Coulter).

RESULTS

In vitro kinetics of ribozyme cleavage

The hairpin ribozyme used here (Fig. 2) has been modified from the original hairpin motif to extend Helix 4 and replace loop 3 with the common RNA tetraloop sequence 5′GGAC (UUCG) GUCC3′ (Anderson *et al.*, 1994). The length of helix 1 was optimized by varying its length and comparing cleavage rates (data not shown). It was found that optimal cleavage (Fig. 1) occurred when helix 1 was 7 bp as shown in Fig. 2. Helix 2 is fixed at 4 bp according to the target selection rules for the hairpin ribozyme.

In vitro characterization of this ribozyme showed high cleavage efficiency. For the kinetic analysis, the ribozyme concentration was limiting, the substrate was present in excess, and reaction times were such that reaction rates were close to linear. The catalytic efficiency was k_{cat}/K_m = 75 min^{-1} μM^{-1}. The individual kinetic parameters were measured to be: K_m = 6.7 nM and k_{cat} = 0.5 min^{-1} (Table 1). This ribozyme has the highest catalytic efficiency reported yet for a hairpin ribozyme.

Long term ribozyme expression in T-cells

Expression of the ribozyme gene in the stably transduced Human Jurkat cell lines was examined by PCR combined with RT, using specific Pol ribozyme primers. Figure 3B shows the results of ribozyme expression in Jurkat cells 5 months (approximately 20 passages) after transduction with the retroviral vectors carrying the Pol

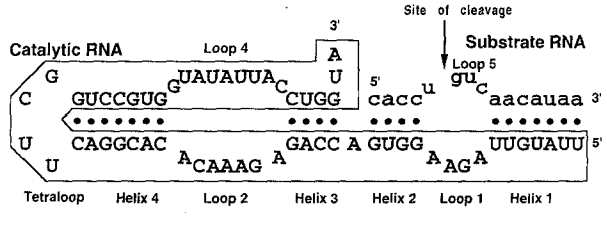

FIG. 2. The HIV-1 pol specific hairpin ribozyme.

TABLE 1

COMPARISON OF CATALYTIC EFFICIENCY OF HAIRPIN RIBOZYMES

Target	K_m (nM)	k_{cat} (min^{-1})	Catalytic efficiency[a]
Native UGACA*GUCCUGUUU	30	2.1	70
HIV-1 pol/tetraloop 2490–2504 CACCU*GUCAACAUAA	6.7	0.5	75
HIV-1 Pol 2490–2504 CACCU*GUCAACAUAA	42	0.2	5
HIV-1 5′ leader 561–576 UGCCC*GUCUGUUGUGU	100	1.6	16

Note. Catalytic parameters of the pol ribozyme, both with or without the tetraloop modification, were determined as described under materials and methods. These were compared to those of the native sequence (Hampel and Tritz, 1989) and the HIV-1 5′ leader ribozyme (Ojwang *et al.*, 1992). The target sequence of each ribozyme is shown.

[a] k_{cat}/K_m (min^{-1} μM^{-1}).

ribozyme. A 60-bp RT-PCR amplified product was detected in both MY-1 and MM6 retroviral vector-transduced Jurkat cells (Fig. 3B, lanes 2 and 8, respectively). This Pol ribozyme-specific RNA product was, however, not detected in Jurkat cells transduced by control vectors, which include the vector alone (LNL6) and a HIV-1 leader sequence ribozyme vector (MJT; Yu *et al.*, 1994b). Furthermore, the product was derived from RNA, not DNA, since no amplified product was observed in the absence of RT in the PCR process (lanes 1, 3, 5, and 7). There was no deleterious effect of long term expression of the ribozyme on cell proliferation as evaluated and compared by cell growth and cell viability (data not shown).

Inhibition of HIV-1 replication by the Pol ribozyme

We investigated the HIV-1 inhibitory effects of the Pol ribozyme in Jurkat and Molt 4/8 cells transduced with MM6 and MY-1 (see Materials and Methods). LNL6 vector-transduced Jurkat cells were used as a negative control. As shown in Fig. 4, the expression of p24 antigen in the culture supernatant of cells infected by the HIV-1 HXB2 strain was reduced 85% in JMY-1 and 99% in JMM6 up to Day 7 in comparison with the control (JLNL6). Expression of p24 in HXB2-infected JMM6 cells was reduced to background levels up to Day 12 postinfection, while HIV-1 P24 expression broke through in HXB2-infected JMY-1 cells. The relatively higher efficiency of virus inhibition of the MM-6 ribozyme vector may be attributable to the higher level of expression and/or greater stability of the pol III-driven transcript (see Fig. 2B) as well as to other factors such as secondary structural and cellular compartmentalization. As a positive control for the challenge experiments, we included Jurkat and Molt 4/8 cells transduced by MJT, which expresses the HIV-1 leader sequence ribozyme as previously published (Yamada *et al.*, 1994). As shown in Fig. 4A, a greater reduction in p24 expression was observed in cells transduced by MM6 than those transduced by MJT. Similar results in the gradation of antiviral efficacy (i.e., MM6 > MJT > MY −1 > LNL6) was observed when transduced Molt 4/8 cells were challenged with virus (Fig. 4B). To investigate the ability of JMM-6 to resist the replication of different HIV-1 strain and the ability of longer term resistance, we further challenged JLNL6 and JMM6 with HIV-1 MN strain (Fig. 5). In this experiment, expression of p24 in MN-infected JMM6 cells was reduced to background levels up to 5 weeks postinfection. The reduction of p24 level in JLNL6 cells after 4 weeks was caused by extensive cell death due to the CPE of the HIV-infected cells as we and others frequently observed for long term-infected cultures.

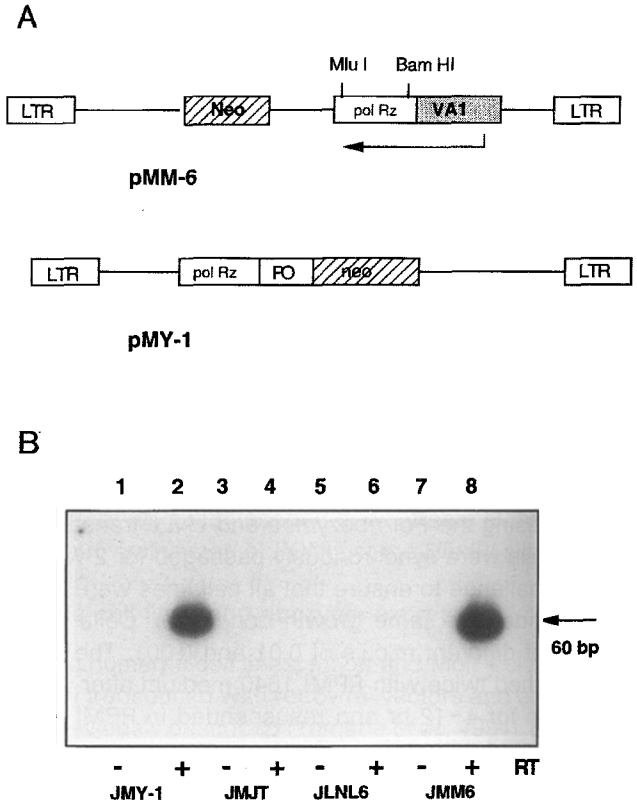

FIG. 3. (A) Schematic representation of the retroviral vector constructs. For pMM-6, two restriction enzyme sites are indicated which were used to replace the leader sequence ribozyme (Yu *et al.*, 1994b) by the pol ribozyme. VA1, adenovirus VA1 promoter. (B) Expression of the ribozyme in stable cell lines. The expression of ribozyme RNA was examined by PCR combined with reverse transcription. −RT, PCR without reverse transcriptase. Each Jurkat cell line used is indicated under the corresponding lanes. The size of the amplified product is marked.

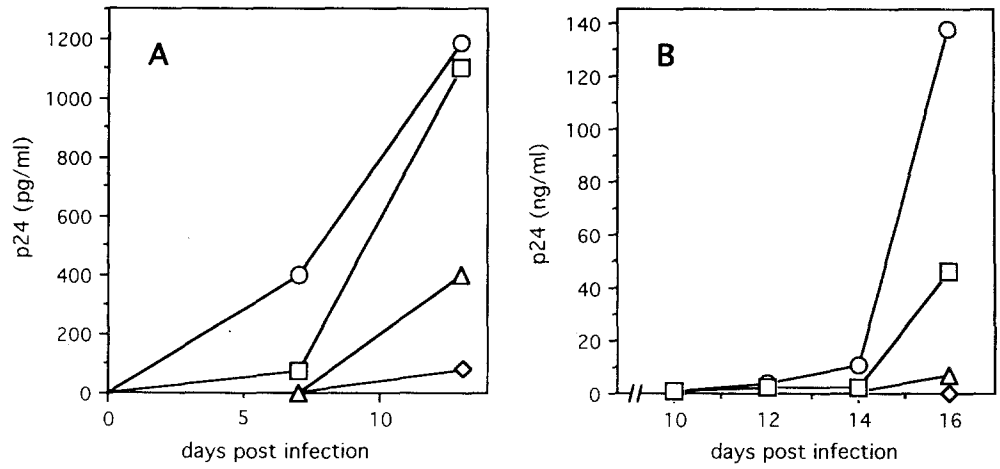

FIG. 4. Inhibition of HIV-1 HXB2 replication by the pol ribozyme. Jurkat (A) and Molt4/8 (B) cell lines stably transduced with LNL-6 (○), MY-1 (□), MJT (△), and MM-6 (◇) were challenged with HXB2 at m.o.i. of 0.01. Virus production was monitored by p24 antigen level.

DISCUSSION

In our previous preclinical studies, we demonstrated the potential of a hairpin ribozyme for anti-HIV gene therapy (Yu *et al.*, 1993; Yamada *et al.*, 1994). Our subsequent efforts have been directed at increasing the catalytic efficiency of ribozymes *in vivo,* as well as exploring the use of alternative targets and developing multitarget ribozyme vectors. We describe here the designing and optimization by *in vitro* cleavage of a modified hairpin ribozyme which showed unprecedented enzyme kinetics *in vitro.* This ribozyme targets a conserved region in the pol gene of HIV-1 (Fig. 1) and confers resistance to virus infection when stably transduced into a human T-cell line. The modification was that the standard GUU loop 3 sequence of the hairpin ribozyme (Hampel *et al.*, 1990; Ojwang *et al.*, 1992) was replaced by a 12-nt tetraloop sequence, 5′GGAC (UUCG) GUCC3′, commonly found in cellular RNA structures. The resulting tetraloop ribozyme has a 7-bp helix 4 (only 3 bp in the conventional hairpin ribozyme) and a new UUCG sequence in loop 3. The tetraloop forms a very stable structure; only one nucleotide in the loop is exposed (Cheong *et al.*, 1990). Therefore, the new structure was added to simultaneously enhance stability of the ribozyme and decrease the size of the exposed loop 3.

The target for this ribozyme was a highly conserved region in the pol gene of HIV-1. In comparing sequences in this region from geographically diverse isolates of HIV-1, only one variant, ELI, was found to contain a mutation, a single base change A → G in position 2492. There is a mismatch for the leader sequence ribozyme in MN strain (see Yu *et al.*, 1993); however, the combination of these two ribozymes will cover the entire HIV-1 spectrum. The catalytic efficiency, k_{cat}/K_m, of 75 $\mu M^{-1}min^{-1}$ for the pol-specific hairpin ribozyme is the highest we have observed for any hairpin ribozyme including the native ribozyme (Table 1). Its catalytic efficiency is 15 times that of the pol ribozyme without the tetraloop modification and 4.7 times that of the 5′ leader ribozyme (Ojwang *et al.*, 1992), which we previously showed to be highly efficient in reducing expression of viral proteins and inhibition of proviral DNA synthesis (Yu *et al.*, 1993, 1994b; Yamada *et al.*, 1994; Leavitt *et al.*, 1994). The persistent ribozyme expression in a human T-cell line (Fig. 3B) indicates that long-term stable expression is possible. As observed previously, we found that protection of human T-cells from different strains of HIV-1 infection was more extensive when the ribozyme gene was expressed from a pol III promoter as opposed to a pol II promoter (Fig. 4; Yu *et al.*, 1993; Yamada *et al.*, 1994). However, the pMY-1 was not used here for direct comparison of promoter strength; rather, it was designed as a first step towards the construction of multitarget ribozyme vectors and testing of the internal ribosome entry site (Fig. 3B; "PO"). The

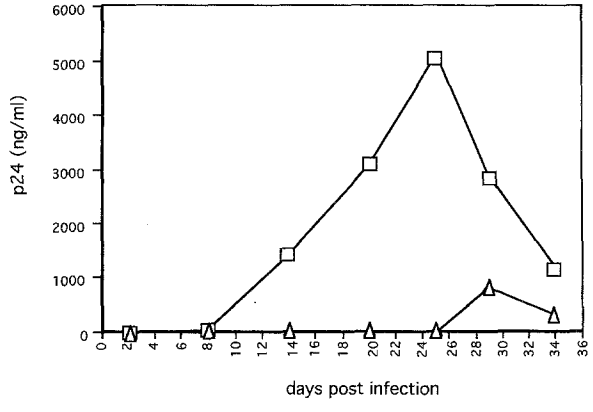

FIG. 5. Long term inhibition of HIV-1 MN replication by the pol ribozyme. Jurkat cell lines stably transduced with LNL-6 (□) and MM-6 (△) were challenged with MN at m.o.i. of 0.001. Virus production was monitored by p24 antigen level.

successful establishment of the stable cell lines, JMY-1 and MMY-1, suggests that the neoprotein can be synthesized by internal initiation of translation.

The immediate future experiments include expression of two ribozymes (leader sequence and pol) simultaneously in the hope of delivering a "one-two punch" against HIV-1. This may be an especially promising approach since the two ribozymes act entirely independently and have different kinetic parameters. The pol ribozyme has a high substrate affinity with low substrate concentration requirement while the leader sequence ribozyme requires higher substrate concentration, but has faster cleavage rate (Table 1). Therefore, the former could serve as the "house cleaner" when the viral targets are lowered by the active cleavage of the leader sequence ribozyme. We have already developed several different multitarget ribozyme retroviral vectors, and functional tests are in progress.

The potential for mutational escape is a potential drawback to any antiviral strategy. HIV-1 has a particularly high mutation rate, a property already well documented to result in, for example, AZT resistant viruses. We seek to diminish the likelihood of mutational escape by targeting highly conserved regions and by combining several targets or approaches. We have, in this study, characterized a second functional hairpin ribozyme that has been modified for increased activity and which recognizes a different target sequence from that previously described. A repertoire of independently targeted hairpin ribozymes may allow a combination strategy able to efficiently block virus replication and emergence of resistant mutants.

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